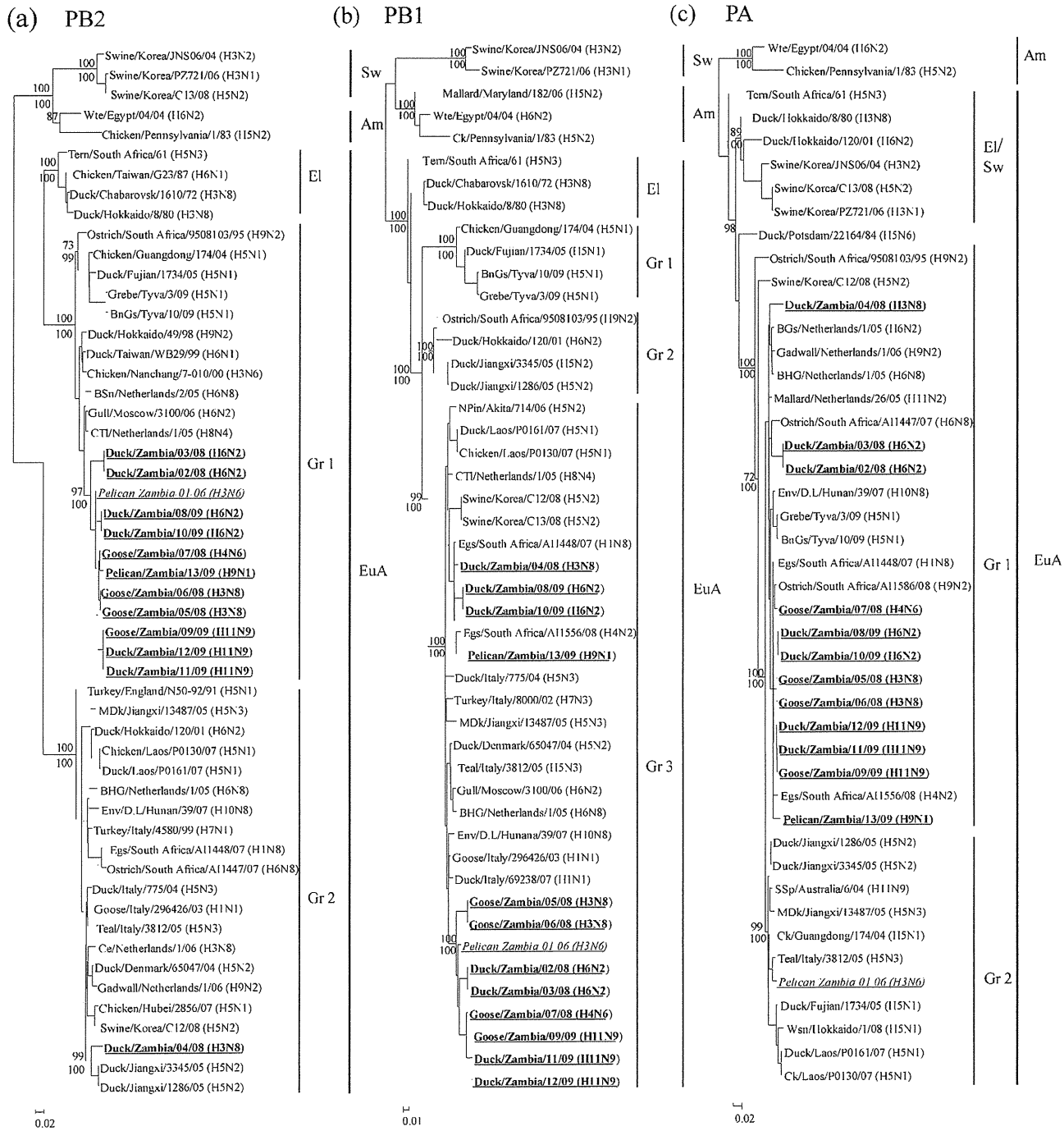

Fig. 3. Phylogenetic relationships of the PB2 (a), PB1 (b) and PA (c) genes of AIVs isolated from wild birds in Zambia. Analysis was based on nt 56–2285 (2230 bp) of PB2, 64–2281 (2218 bp) of PB1 and 30–2098 (2069 bp) of PA. Numbers above and below branch nodes indicate neighbour-joining bootstrap values of ⩾50 % and Bayesian posterior probabilities of >95 %, respectively. Due to space limitations, not all supports are indicated. Virus strains characterized in the present study are in bold and underlined, whilst the first AIV isolate in Zambia is italicized and underlined. Bars, number of substitutions per site. Lineages: El/Sw, early/swine; Sw, swine. Strain names: BGs, barnacle goose; BnGs, bean goose; Ce, common eider; Ck, chicken; CTl, common teal; D.L, Dongting Lake; Env, environment; MDk, migratory duck; SSp, sharp-tailed sandpiper; Wsn, whooper swan. Other abbreviations are listed in the legends of Figs 1 and 2.

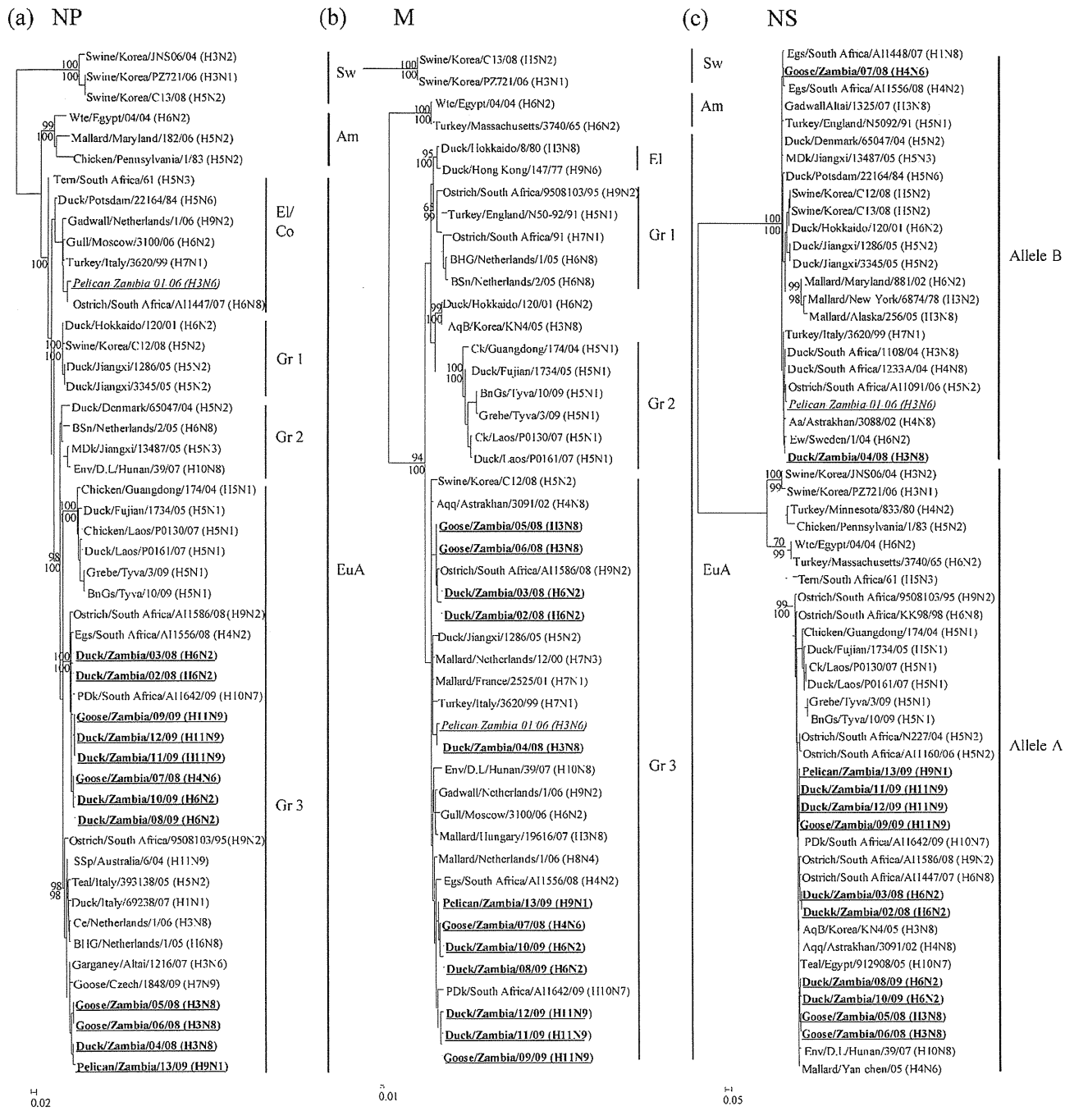

Fig. 4. Phylogenetic relationships of the NP (a), M (b) and NS (c) genes of AIVs isolated from wild birds in Zambia. Analysis was based on nt 46–1489 (1444 bp) of NP, 32–753 (722 bp) of M and 57–705 (649 bp) of NS. Numbers above and below the branch nodes indicate neighbour-joining bootstrap values of $\geqslant$50 % and Bayesian posterior probabilities of >95, respectively. Owing to space limitations, not all supports are indicated. Virus strains characterized in the present study are in bold and underlined, whilst the first influenza virus isolate from an avian host in Zambia is italicized. Bars, number of substitutions per site. Lineages: El/Co, early/contemporary. Strain names: Aa, *Anas angustirostris*; AqB, aquatic bird; Aqq, *Anas querquedula*. Other abbreviations are listed in the legends of Figs 1–3.

Phylogenetic analysis of the non-structural (NS) gene indicated that the NS genes of ten of the viruses from wild birds in Zambia comprised the A allele, whilst the other three were of the B allele (Fig. 4c). Some of the NS genes were closely related to viruses isolated mainly in Asia and Africa, particularly those isolated in South Africa (Fig. 4c).

The NS gene tree clearly demonstrated that, among the viruses examined, the A allele was predominant and that two genetically distinct gene pools, corresponding to NS alleles A and B, were co-circulating in wild birds in this region during the surveillance period.

Amino acid sequence analysis

Although it is difficult to ascertain the capacity of a non-pathogenic AIV/LPAIV from wild waterfowl to cause interspecies transmission into other animals, close monitoring of host-associated signatures in viral proteins may provide some clues regarding an isolate's zoonotic potential. Several amino acids that are preferentially associated with human influenza viruses have been described (Chen *et al.*, 2006; Finkelstein *et al.*, 2007; Shaw *et al.*, 2002). We examined the deduced amino acid sequences of all the internal proteins of all the wild-bird isolates from Zambia and identified some human-associated amino acids in the genome of some strains (Table 2). Zb08 (H6N2) and Zb10 (H6N2) possessed the human-associated amino acid methionine at position 475 of the PB2 protein, which has been described to be 100 % conserved in the influenza viruses that caused the 1918, 1957 and 1968 human pandemics (Finkelstein *et al.*, 2007). Zb13 (H9N1) had a serine at position 66 of the PB1-F2 polypeptide, which was shown previously to contribute to increased virulence in mice (Conenello *et al.*, 2007). Zb13 (H9N1) also possessed the human-associated amino acid alanine at position 76 of the PB1-F2 protein. Six isolates were found to have the human-associated amino acid serine at position 82 of the PB1-F2 protein, whilst only Zb08 (H6N2) and Zb10 (H6N2) had the human-associated amino acid glycine at position 87 of this polypeptide. In the M2 protein, Zb04 (H3N8) possessed the human-associated amino acid valine at position 28. At position 55 of the M2 protein, Zb07 (H4N6), Zb08 (H6N2), Zb10 (H6N2) and Zb12 (H11N9) were found to possess the human-associated amino acid phenylalanine. It is noteworthy that, although the human-associated amino acids found in some of the virus isolates analysed in this report are not unique to these isolates, these residues are rarely found among AIVs isolated from members of the orders Anseriformes and Charadriiformes (our unpublished data).

Table 2. Human-associated amino acids identified in viral proteins of AIVs isolated in Zambia

Protein	Aa position*	Host		Isolate†
		Avian	Human	
PB2	475	L	M	Zb08 (H6N2)
				Zb10 (H6N2)
PB1-F2	66	N	S‡	Zb13 (H9N1)
	76	V	A	Zb13 (H9N1)
	82	L	S	Zb04 (H3N8)
				Zb05 (H3N8)
				Zb06 (H3N8)
				Zb08 (H6N2)
				Zb10 (H6N2)
				Zb13 (H9N1)
	87	E	G	Zb08 (H6N2)
				Zb10 (H6N2)
M2	28	I	V	Zb04 (H3N8)
	55	L	F	Zb07 (H4N6)
				Zb08 (H6N2)
				Zb10 (H6N2)
				Zb12 (H11N9)

*For references of human-associated residues at these specific positions, see Chen *et al.* (2006), Finkelstein *et al.* (2007) and Shaw *et al.* (2002).

†Names of isolates possessing human-associated amino acid residues.

‡The amino acid serine at position 66 of the PB1-F2 protein is not a human-associated residue but was shown previously to increase virulence in mice (Conenello *et al.*, 2007).

Replication and pathogenicity of selected viruses in mice

Amino acid sequence analysis revealed that several isolates from wild birds in Zambia had human-associated residues in their genome (Table 2). Therefore, we sought to investigate whether there could be a difference in virus replication and/or pathogenicity in a mammalian host between viruses either possessing or lacking human-associated residues. For this purpose, we compared the replication ability and pathogenicity of two isolates, Zb03 (H6N2) and Zb10 (H6N2), in mice. Four human-associated residues were identified in some viral proteins of Zb10 (H6N2), whilst none was observed in the genome of Zb03 (H6N2) (Table 2). We also tested the replication capacity and pathogenicity of Zb04 (H3N8) in mice, because it had two human-associated residues in its genome and was of a subtype distinct from that of Zb10 (H6N2).

All the tested viruses replicated in the lungs of mice without prior adaptation, with virus titres ranging from $10^{3.3}$ to $10^{4.8}$ EID_{50} g^{-1} (Table 3). None of the viruses was detected in the brain. It was noted that mice inoculated with Zb10 (H6N2) showed higher virus titres that were statistically significantly different from those of Zb03 (H6N2)-infected mice (Table 3). Virus was detected in the lungs of all five mice inoculated with Zb04 (H3N8) and Zb10 (H6N2), whilst, in Zb03 (H6N2)-inoculated mice, virus was detected in three of the five mice.

Mice infected with Zb10 (H6N2) exhibited more weight loss and delayed weight gain [weight returned to baseline after day 7 post-inoculation (p.i.)] than those inoculated with Zb03 (H6N2) (Fig. 5). Zb04 (H3N8)-inoculated mice showed significant weight loss early on p.i. when compared with Zb03 (H6N2)- or mock-inoculated control mice (Fig. 5). Mild to considerable ruffled fur was noted between days 1 and 3 p.i. in mice infected with Zb10 (H6N2) and Zb04 (H3N8) but not in Zb03 (H6N2)-inoculated mice.

Table 3. Replication of selected AIVs isolated from wild waterfowl in Zambia in BALB/c mice

Virus	No. positive/ total	Mean virus titre of positive samples (log_{10} EID_{50} g^{-1})		*P* value
		Lung	Brain	
Zb03 (H6N2)*	3/5	3.3	$<10^{1.5}$	–
Zb04 (H3N8)†	5/5	3.6	$<10^{1.5}$	0.099
Zb10 (H6N2)†	5/5	4.8	$<10^{1.5}$	0.001‡

*Virus with no apparent human/mammalian-associated residues in its genome.
†Viruses with human-associated residues in their genome.
‡Virus titre in the lungs of mice inoculated with Zb10 (H6N2) was significantly higher than that of Zb03 (H6N2)-inoculated mice (Student's *t*-test, $P<0.05$).

All the mice survived the infection for the 14-day observation period.

DISCUSSION

In this study, we genetically and biologically characterized AIVs isolated from wild birds in Zambia. During the surveillance period, AIVs were isolated mainly between June and November, a time frame encompassing the period when palearctic migrants are absent or rare, as well as when they are present. Palearctic birds usually start to arrive in Zambia between September and December and leave between January and May. Our isolation of AIVs between June and August of 2008 and 2009 when palearctic migrants were scarce raises the possibility of yearly persistence of AIVs in indigenous waterfowl in southern Africa. This idea is further supported by our phylogenetic analyses, which showed the separate clustering of southern African isolates, with the glycoprotein genes of H11N9 viruses characterized in this report forming a distinct sublineage within the Eurasian lineage (Fig. 3a, b and Supplementary Figs S1c and S2c). In neighbouring Zimbabwe, AIVs were also detected in Afro-tropical waterfowl in periods when palearctic birds were rare (Caron *et al.*, 2010). Moreover, AIVs were detected from Afro-tropical bird species in several major wetlands in Africa (Gaidet *et al.*, 2007). These data not only support the notion of a possible endemicity of AIVs in Afro-tropical ecosystems where high temperatures experienced in these regions may restrict the persistence and transmissibility of AIVs (Brown *et al.*, 2009), but also raise the possibility that palearctic migrants may also carry AIVs from Africa into Eurasia. However, the extent to which Afro-tropical ecosystems depend on introductions of AIVs by Eurasian migrants to sustain the possible endemic state remains to be clarified.

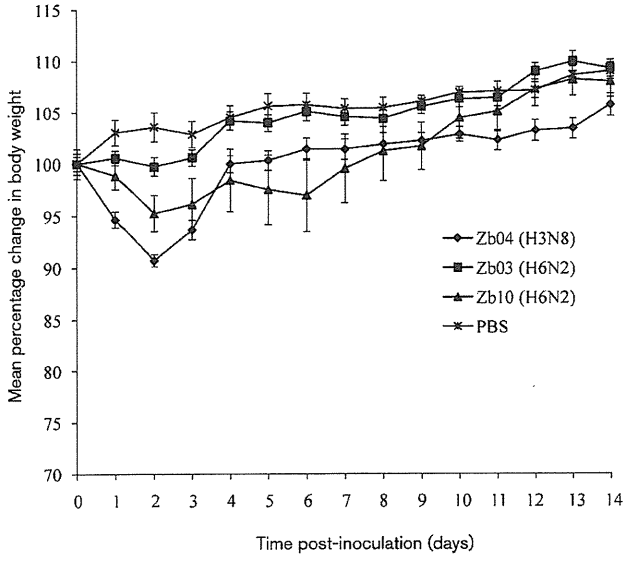

Fig. 5. Weight loss in mice inoculated with selected AIVs from wild waterfowl in Zambia. Data are presented as mean body weight change per group ± SD. Statistically significant weight loss (Student's *t*-test, $P<0.05$) was observed in Zb04 (H3N8)-inoculated mice at days 1 ($P=0.03$), 2 ($P=0.01$) and 3 ($P=0.03$) p.i. compared with mock-inoculated control mice.

The detection of five distinct HA and NA subtypes suggested that a variety of subtypes could be circulating in wild birds in this region. Whilst 11 of the isolates were detected in wild ducks and geese, confirming the major role of these birds in the perpetuation of AIVs (Olsen *et al.*, 2006; Webster *et al.*, 1992), Zb13 (H9N1) was isolated from an atypical avian host, a great white pelican. Despite several AIV surveillance studies that involved sampling from the Pelecaniformes worldwide (Gaidet *et al.*, 2007; Munster *et al.*, 2007; Olsen *et al.*, 2006), the number of AIVs detected from this order has remained low. Thus, we consider the two instances in which we isolated AIVs from these birds as incidental findings, but we do not exclude the possibility that white pelicans, which are native to southern Africa, may also play a major role in influenza virus ecology in this region.

Phylogenetic analyses demonstrated that all the gene segments of the viruses reported in this study clustered with contemporary viruses of the Eurasian avian lineage. Most genes were closely related to those of AIVs isolated from wild and domestic birds in South Africa. AIVs originating in wild birds have been implicated in avian influenza outbreaks in farmed birds in South Africa with serious economic consequences (Abolnik, 2007; Abolnik *et al.*, 2007, 2010; Alexander, 2007; Brown, 2010). These data highlight the need for continued monitoring of AIVs in wild and domestic birds in southern Africa for avian influenza control. It is also important to clarify the extent of influenza virus exchange between wild birds and domesticated birds (including ostriches) in the region, as

results from a study in China demonstrated that a two-way transmission of influenza viruses between terrestrial and aquatic birds may increase opportunities for the generation of reassortant viruses with pandemic potential (Li *et al.*, 2003). Furthermore, the potential role of human related activities (e.g. the poultry trade) in AIV dissemination should not be ignored.

A number of human-associated amino acids were observed in some viral proteins of some viruses tested. The two possible means by which AIVs may acquire 'novel' amino acids are either through genetic reassortment or through point mutations. Genetic analyses of AIVs isolated from wild and terrestrial birds in southern Africa have demonstrated the involvement of ostriches in the evolution and epidemiology of AIVs in this region (Abolnik, 2007; Abolnik *et al.*, 2007, 2010; this study). Recently, Shinya *et al.* (2009) demonstrated that ostriches may be involved in the emergence of viruses possessing mammalian-associated amino acids lysine and asparagine at positions 627 and 701 of the PB2 protein, respectively. Indeed, an examination of PB2 gene sequences of viruses isolated from ostriches in South Africa between 1995 and 2008 showed that four viruses had lysine and one virus possessed asparagine at positions 627 and 701 of the PB2 protein, respectively (data not shown). Therefore, if a two-way transmission of AIVs between ostriches and wild aquatic birds in southern Africa exists, these data indicate that the human-associated amino acids observed in some internal proteins of some of the isolates examined here may have been acquired through genetic reassortment with viruses from ostriches. Unfortunately, the lack of complete internal protein gene sequences of isolates from ostriches in South Africa for a comprehensive study makes it difficult to reach this conclusion. Moreover, genetic analyses of the deduced amino acids revealed that the surface proteins of the viruses listed in Table 2 maintained typical features of non-pathogenic wild waterfowl isolates, including conservation of putative glycosylation sites and no NA stalk deletions, and did not exhibit evidence for accelerated or increased amino acid substitutions, suggesting that these viruses may not have circulated extensively in land-based avian species. These observations leave open the possibility that the human-associated amino acids in the viral proteins of some isolates from Zambia may have been acquired in wild waterfowl or other non-gallinaceous birds. Whether some African waterfowl may provide an environment that may lead to the selection of AIVs with human/mammalian-associated amino acids is a question deserving further exploration.

In a mouse model, we demonstrated that all the tested viruses replicated in mouse lung without prior adaptation and that mice infected with isolates having human-associated residues displayed increased virus titres and caused increased morbidity, as measured by weight loss, than those inoculated with Zb03 (H6N2). Although it is tempting to conclude that possession of human-associated residues may have impacted on virus replication and pathogenicity in mice, there is need for caution, because the influence of other residues was not ruled out in the current study. In fact, there were 68 amino acid differences in viral proteins between Zb03 (H6N2) and Zb10 (H6N2). Therefore, investigations employing reverse genetics and site-directed mutagenesis may be needed to explain more fully the observed differences. To our knowledge, the present study is the first to demonstrate the ability of non-HPAIVs from wild birds in Africa to replicate without adaptation and cause illness in a mammalian host. Elsewhere, although few in number, AIVs from wild birds of considerable numbers of HA subtypes have been shown to replicate in mice and ferrets without adaptation, causing varied degrees of morbidity (Driskell *et al.*, 2010; Gillim-Ross *et al.*, 2008; Joseph *et al.*, 2007; Kim *et al.*, 2010; Wan *et al.*, 2008). These studies have highlighted the potential risk of direct transmission of non-HPAIVs from wild birds to mammalian species. Whilst direct transmission of AIVs from wild birds to humans has not been reported, serological evidence of AIV infection in three persons with substantial exposure to wild waterfowl and game birds argues for a possible direct transmission of AIVs from wild birds to humans (Gill *et al.*, 2006). Moreover, both natural and experimental infections of humans with AIVs, together with serological data, have emphasized the susceptibility of humans to several AIV subtypes (Myers *et al.*, 2007; Peiris *et al.*, 2007; Shortridge, 1992). Thus, the potential threat posed to both animal and public health by some of the viruses characterized currently cannot be overemphasized.

Here, we demonstrated that the 12 influenza viruses isolated from wild waterfowl in Zambia belonged to the contemporary Eurasian avian lineage. We have shown the possibility that AIVs could persist in wild waterfowl in a Zambian ecosystem, with transmission of viruses involving wild and domestic avian species in southern Africa, Europe and Asia. This study further established that some AIVs from wild waterfowl in Zambia may have the potential to infect mice directly without adaptation. Overall, the present study raises concerns for continued monitoring of AIVs in wild and domestic birds in southern Africa and suggests that complete characterization of isolates may help in the identification of strains that may have potential for future incursions into humans and other animals.

METHODS

Viruses and sequencing. The viruses characterized in the present study were isolated from wild waterfowl faecal specimens collected in Lochinvar National Park between April 2008 and November 2009 (Table 1). All virus isolation was performed using 10–11-day-old embryonated chicken's eggs. The isolates were subtyped by standard HA inhibition and NA inhibition tests, as well as by sequencing of the HA and NA genes. The viruses were passaged once in eggs before being used in this study. Viral RNA extraction, cDNA synthesis, PCR and sequencing were carried out as described previously (Simulundu *et al.*, 2009).

Phylogenetic analyses. Phylogenetic trees were constructed by the neighbour-joining bootstrap method with 1000 replicates applied

using MEGA4 (Tamura *et al.*, 2007). The gene tree topologies obtained in MEGA4 were then confirmed using Bayesian methods implemented in MRBAYES version 3.1.2 (Huelsenbeck & Ronquist, 2001). Specifically, we used the program ModelTest version 3.7 (Posada & Crandall, 2001), applied in PAUP* version 4.0 (Swofford, 2001), to determine the appropriate evolutionary model that best fitted the data. The HA, NA, PB2, PB1, PA and NP nucleotide sequence data were best fitted by the general time reversible plus invariant sites plus gamma-distributed (GTR+I+G) model, whilst the Hasegawa–Kishino–Yano (plus invariant sites) plus gamma-distributed models (HKY+G and HKY+I+G) were preferred for the NS and M sequence data, respectively. In Bayesian analysis, we used one to four replicates of 1 million generations, with four chains sampled every 100 generations. All replicates converged with less than 0.01 SD of split frequencies.

Experimental infection of mice. Groups of 6-week-old BALB/c mice (ten mice per group) were lightly anaesthetized with isoflurane and inoculated intranasally with 0.05 ml virus-infected chorioallantoic fluid containing Zb03 (H6N2), Zb04 (H3N8) or Zb10 (H6N2) ($10^{7.5}$ EID_{50} ml^{-1}). To serve as a control, a group of five mice was mock infected with sterile PBS. Mice were observed daily for morbidity (weight loss, ruffled fur and hunching) and mortality for 14 days. On day 3 p.i., half of the virus-inoculated mice were euthanized, and the titres of virus in the lung and brain were determined using eggs. Briefly, a 10% lung and brain tissue homogenate was prepared using minimal essential medium (Gibco) containing antibiotics. The tissue homogenates were clarified by centrifugation and titrated in 10–11-day-old embryonated chicken's eggs. The virus titre was calculated as the $\log_{10}$ EID_{50} $(g\ tissue)^{-1}$ by the method of Reed & Muench (1938).

ACKNOWLEDGEMENTS

We thank the Zambia Wildlife Authority for supporting the wild-bird influenza A virus surveillance programme in Zambia. We are also grateful to H. Miyamoto, A. Ohnuma and A. Yokoyama for excellent technical assistance. This work was supported by the Program of Founding Research Centers for Emerging and Reemerging Infectious Diseases and the Global COE Program 'Establishment of International Collaboration Centers for Zoonosis Control' from the Ministry of Education, Culture, Sports, Science and Technology (MEXT), Japan.

REFERENCES

Abolnik, C. (2007). Molecular characterization of H5N2 avian influenza viruses isolated from South African ostriches in 2006. *Avian Dis* **51**, 873–879.

Abolnik, C., Bisschop, S., Gerdes, T., Olivier, A. & Horner, R. (2007). Outbreaks of avian influenza H6N2 viruses in chickens arose by a reassortment of H6N8 and H9N2 ostrich viruses. *Virus Genes* **34**, 37–45.

Abolnik, C., Gerdes, G. H., Sinclair, M., Ganzevoort, B. W., Kitching, J. P., Burger, C. E., Romito, M., Dreyer, M., Swanepoel, S. & other authors (2010). Phylogenetic analysis of influenza A viruses (H6N8, H1N8, H4N2, H9N2, H10N7) isolated from wild birds, ducks, and ostriches in South Africa from 2007 to 2009. *Avian Dis* **54** (Suppl.), 313–322.

Alexander, D. J. (2007). Summary of avian influenza activity in Europe, Asia, Africa, and Australasia, 2002-2006. *Avian Dis* **51** (Suppl.), 161–166.

Bahl, J., Vijaykrishna, D., Holmes, E. C., Smith, G. J. D. & Guan, Y. (2009). Gene flow and competitive exclusion of avian influenza A virus in natural reservoir hosts. *Virology* **390**, 289–297.

Beare, A. S. & Webster, R. G. (1991). Replication of avian influenza viruses in humans. *Arch Virol* **119**, 37–42.

Brown, I. H. (2010). Summary of avian influenza activity in Europe, Asia, and Africa, 2006–2009. *Avian Dis* **54** (Suppl.), 187–193.

Brown, J. D., Goekjian, G., Poulson, R., Valeika, S. & Stallknecht, D. E. (2009). Avian influenza virus in water: infectivity is dependent on pH, salinity and temperature. *Vet Microbiol* **136**, 20–26.

Capua, I. & Alexander, D. J. (2006). The challenge of avian influenza to the veterinary community. *Avian Pathol* **35**, 189–205.

Caron, A., Abolnik, C., Mundava, J., Gaidet, N., Burger, C. E., Mochotlhoane, B., Bruinzeel, L., Chiweshe, N., de Garine-Wichatitsky, M. & Cumming, G. S. (2010). Persistence of low pathogenic avian influenza virus in waterfowl in a Southern African ecosystem. *EcoHealth* (Epub ahead of print).

Chen, G.-W., Chang, S.-C., Mok, C.-K., Lo, Y.-L., Kung, Y.-N., Huang, J.-H., Shih, Y.-H., Wang, J.-Y., Chiang, C. & other authors (2006). Genomic signatures of human versus avian influenza A viruses. *Emerg Infect Dis* **12**, 1353–1360.

Conenello, G. M., Zamarin, D., Perrone, L. A., Tumpey, T. & Palese, P. (2007). A single mutation in the PB1-F2 of H5N1 (HK/97) and 1918 influenza A viruses contributes to increased virulence. *PLoS Pathog* **3**, 1414–1421.

de Wit, E., Kawaoka, Y., de Jong, M. D. & Fouchier, R. A. (2008). Pathogenicity of highly pathogenic avian influenza virus in mammals. *Vaccine* **26** (Suppl. 4), D54–D58.

Driskell, E. A., Jones, C. A., Stallknecht, D. E., Howerth, E. W. & Tompkins, S. M. (2010). Avian influenza virus isolates from wild birds replicate and cause disease in a mouse model of infection. *Virology* **399**, 280–289.

Duan, L., Campitelli, L., Fan, X. H., Leung, Y. H., Vijaykrishna, D., Zhang, J. X., Donatelli, I., Delogu, M., Li, K. S. & other authors (2007). Characterization of low-pathogenic H5 subtype influenza viruses from Eurasia: implications for the origin of highly pathogenic H5N1 viruses. *J Virol* **81**, 7529–7539.

Ducatez, M. F., Olinger, C. M., Owoade, A. A., De Landtsheer, S., Ammerlaan, W., Niesters, H. G. M., Osterhaus, A. D. M. E., Fouchier, R. A. M. & Muller, C. P. (2006). Avian flu: multiple introductions of H5N1 in Nigeria. *Nature* **442**, 37.

Finkelstein, D. B., Mukatira, S., Mehta, P. K., Obenauer, J. C., Su, X., Webster, R. G. & Naeve, C. W. (2007). Persistent host markers in pandemic and H5N1 influenza viruses. *J Virol* **81**, 10292–10299.

Gaidet, N., Dodman, T., Caron, A., Balança, G., Desvaux, S., Goutard, F., Cattoli, G., Lamarque, F., Hagemeijer, W. & Monicat, F. (2007). Avian influenza viruses in water birds, Africa. *Emerg Infect Dis* **13**, 626–629.

Gaidet, N., Cattoli, G., Hammoumi, S., Newman, S. H., Hagemeijer, W., Takekawa, J. Y., Cappelle, J., Dodman, T., Joannis, T. & other authors (2008). Evidence of infection by H5N2 highly pathogenic avian influenza viruses in healthy wild waterfowl. *PLoS Pathog* **4**, e1000127.

Gill, J. S., Webby, R., Gilchrist, M. J. R. & Gray, G. C. (2006). Avian influenza among waterfowl hunters and wildlife professionals. *Emerg Infect Dis* **12**, 1284–1286.

Gillim-Ross, L., Santos, C., Chen, Z., Aspelund, A., Yang, C.-F., Ye, D., Jin, H., Kemble, G. & Subbarao, K. (2008). Avian influenza H6 viruses productively infect and cause illness in mice and ferrets. *J Virol* **82**, 10854–10863.

Hinshaw, V. S., Webster, R. G., Easterday, B. C. & Bean, W. J., Jr (1981). Replication of avian influenza A viruses in mammals. *Infect Immun* **34**, 354–361.

Huelsenbeck, J. P. & Ronquist, F. R. (2001). MRBAYES: Bayesian inference of phylogenetic trees. *Bioinformatics* **17**, 754–755.

Joseph, T., McAuliffe, J., Lu, B., Jin, H., Kemble, G. & Subbarao, K. (2007). Evaluation of replication and pathogenicity of avian influenza a H7 subtype viruses in a mouse model. *J Virol* **81**, 10558–10566.

Kida, H., Ito, T., Yasuda, J., Shimizu, Y., Itakura, C., Shortridge, K. F., Kawaoka, Y. & Webster, R. G. (1994). Potential for transmission of avian influenza viruses to pigs. *J Gen Virol* **75**, 2183–2188.

Kim, H.-R., Lee, Y.-J., Lee, K.-K., Oem, J.-K., Kim, S.-H., Lee, M. -H., Lee, O.-S. & Park, C.-K. (2010). Genetic relatedness of H6 subtype avian influenza viruses isolated from wild birds and domestic ducks in Korea and their pathogenicity in animals. *J Gen Virol* **91**, 208–219.

Li, K. S., Xu, K. M., Peiris, J. S., Poon, L. L., Yu, K. Z., Yuen, K. Y., Shortridge, K. F., Webster, R. G. & Guan, Y. (2003). Characterization of H9 subtype influenza viruses from the ducks of southern China: a candidate for the next influenza pandemic in humans? *J Virol* **77**, 6988–6994.

Li, K. S., Guan, Y., Wang, J., Smith, G. J. D., Xu, K. M., Duan, L., Rahardjo, A. P., Puthavathana, P., Buranathai, C. & other authors (2004). Genesis of a highly pathogenic and potentially pandemic H5N1 influenza virus in eastern Asia. *Nature* **430**, 209–213.

Munster, V. J., Baas, C., Lexmond, P., Waldenström, J., Wallensten, A., Fransson, T., Rimmelzwaan, G. F., Beyer, W. E., Schutten, M. & other authors (2007). Spatial, temporal, and species variation in prevalence of influenza A viruses in wild migratory birds. *PLoS Pathog* **3**, e61.

Murphy, B. R., Hinshaw, V. S., Sly, D. L., London, W. T., Hosier, N. T., Wood, F. T., Webster, R. G. & Chanock, R. M. (1982). Virulence of avian influenza A viruses for squirrel monkeys. *Infect Immun* **37**, 1119–1126.

Myers, K. P., Setterquist, S. F., Capuano, A. W. & Gray, G. C. (2007). Infection due to 3 avian influenza subtypes in United States veterinarians. *Clin Infect Dis* **45**, 4–9.

Olsen, B., Munster, V. J., Wallensten, A., Waldenström, J., Osterhaus, A. D. & Fouchier, R. A. (2006). Global patterns of influenza A virus in wild birds. *Science* **312**, 384–388.

Peiris, J. S., de Jong, M. D. & Guan, Y. (2007). Avian influenza virus (H5N1): a threat to human health. *Clin Microbiol Rev* **20**, 243–267.

Posada, D. & Crandall, K. A. (2001). Selecting the best-fit model of nucleotide substitution. *Syst Biol* **50**, 580–601.

Reed, L. J. & Muench, H. (1938). A simple method of estimating fifty percent endpoints. *Am J Hyg* **27**, 493–497.

Röhm, C., Horimoto, T., Kawaoka, Y., Süss, J. & Webster, R. G. (1995). Do hemagglutinin genes of highly pathogenic avian influenza viruses constitute unique phylogenetic lineages? *Virology* **209**, 664–670.

Shaw, M., Cooper, L., Xu, X., Thompson, W., Krauss, S., Guan, Y., Zhou, N., Klimov, A., Cox, N. & other authors (2002). Molecular changes associated with the transmission of avian influenza a H5N1 and H9N2 viruses to humans. *J Med Virol* **66**, 107–114.

Shinya, K., Makino, A., Ozawa, M., Kim, J. H., Sakai-Tagawa, Y., Ito, M., Le, Q. M. & Kawaoka, Y. (2009). Ostrich involvement in the selection of H5N1 influenza virus possessing mammalian-type amino acids in the PB2 protein. *J Virol* **83**, 13015–13018.

Shortridge, K. F. (1992). Pandemic influenza: a zoonosis? *Semin Respir Infect* **7**, 11–25.

Simulundu, E., Mweene, A. S., Tomabechi, D., Hang'ombe, B. M., Ishii, A., Suzuki, Y., Nakamura, I., Sawa, H., Sugimoto, C. & other authors (2009). Characterization of H3N6 avian influenza virus isolated from a wild white pelican in Zambia. *Arch Virol* **154**, 1517–1522.

Smith, G. J. D., Fan, X. H., Wang, J., Li, K. S., Qin, K., Zhang, J. X., Vijaykrishna, D., Cheung, C. L., Huang, K. & other authors (2006). Emergence and predominance of an H5N1 influenza variant in China. *Proc Natl Acad Sci U S A* **103**, 16936–16941.

Swofford, D. L. (2001). PAUP*: phylogenetic analysis using parsimony (and other methods) 4.0 beta. Sunderland, MA: Sinauer Associates.

Tamura, K., Dudley, J., Nei, M. & Kumar, S. (2007). MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. *Mol Biol Evol* **24**, 1596–1599.

Wan, H., Sorrell, E. M., Song, H., Hossain, M. J., Ramirez-Nieto, G., Monne, I., Stevens, J., Cattoli, G., Capua, I. & other authors (2008). Replication and transmission of H9N2 influenza viruses in ferrets: evaluation of pandemic potential. *PLoS ONE* **3**, e2923.

Wang, G., Zhan, D., Li, L., Lei, F., Liu, B., Liu, D., Xiao, H., Feng, Y., Li, J. & other authors (2008). H5N1 avian influenza re-emergence of Lake Qinghai: phylogenetic and antigenic analyses of the newly isolated viruses and roles of migratory birds in virus circulation. *J Gen Virol* **89**, 697–702.

Webster, R. G., Bean, W. J., Gorman, O. T., Chambers, T. M. & Kawaoka, Y. (1992). Evolution and ecology of influenza A viruses. *Microbiol Rev* **56**, 152–179.

World Health Organization (2010). Cumulative number of confirmed human cases of avian influenza A/(H5N1) reported to WHO. http://www.who.int/csr/disease/avian_influenza/country/cases_table_2010_12_29/en/index.html. Accessed 4 January 2011.

Xu, K. M., Smith, G. J. D., Bahl, J., Duan, L., Tai, H., Vijaykrishna, D., Wang, J., Zhang, J. X., Li, K. S. & other authors (2007). The genesis and evolution of H9N2 influenza viruses in poultry from southern China, 2000 to 2005. *J Virol* **81**, 10389–10401.

Japanese Journal of Veterinary Research 59(1): 15-22, 2011

FULL PAPER

Virological surveillance and phylogenetic analysis of the PB2 genes of influenza viruses isolated from wild water birds flying from their nesting lakes in Siberia to Hokkaido, Japan in autumn

Rozanah Asmah Abdul Samad[1)], Yoshihiro Sakoda[1, 4)], Yoshimi Tsuda[1)], Edgar Simulundu[3)], Rashid Manzoor[3)], Masatoshi Okamatsu[1, 4)], Kimihito Ito[3)] and Hiroshi Kida[1, 2, 4, 5, *)]

[1)]Laboratory of Microbiology, Department of Disease Control, Graduate School of Veterinary Medicine, Hokkaido University, Sapporo 060-0818, Japan

[2)]Hokkaido University Research Center for Zoonosis Control, Sapporo 001-0020, Japan

[3)]Department of Global Epidemiology, Hokkaido University Research Center for Zoonosis Control, Sapporo 001-0020, Japan

[4)]OIE Reference Laboratory for Highly Pathogenic Avian Influenza, Sapporo 060-0818, Japan

[5)]SORST, Japan Science and Technology Agency (JST), 4-1-8 Honcho Kawaguchi, Saitama 332-0012, Japan

Received for publication, November 8, 2010; accepted, December 7, 2010

Abstract

Recent introduction of H5N1 highly pathogenic avian influenza virus (HPAIV) in wild birds from poultry in Eurasia signaled the possibility that this virus may perpetuate in nature. Surveillance of avian influenza especially in migratory birds, therefore, has been conducted to provide information on the viruses brought by them to Hokkaido, Japan, from their nesting lakes in Siberia in autumn. During 2008-2009, 62 influenza viruses of 21 different combinations of hemagglutinin (HA) and neuraminidase (NA) subtypes were isolated. Up to September 2010, no HPAIV has been found, indicating that H5N1 HPAIV has not perpetuated at least dominantly in the lakes where ducks nest in summer in Siberia. The PB2 genes of 54 influenza viruses out of 283 influenza viruses isolated in Hokkaido in 2000-2009 were phylogenetically analysed. None of the genes showed close relation to those of H5N1 HPAIVs that were detected in wild birds found dead in Eurasia on the way back to their northern territory in spring.

Keywords: *Avian influenza, migratory ducks, PB2 gene, surveillance*

*Corresponding author: Hiroshi Kida, Laboratory of Microbiology, Department of Disease Control, Graduate School of Veterinary Medicine, Hokkaido University, Sapporo 060-0818, Japan
Phone: +81-11-706-5207. Fax: +81-11-706-5273. E-mail: kida@vetmed.hokudai.ac.jp

Introduction

Ecological studies have revealed that a vast influenza virus gene pool for avian and mamalian influenza exists in migratory ducks[10]. Each of the sixteen hemagglutinin (HA) and nine neuramidase (NA) subtypes of influenza A viruses are perpetuated among migratory ducks and their nesting lake water in nature[4,7,15,23]. Transmission of H5 or H7 influenza viruses to domestic birds and especially in chickens may result in the emergence of highly pathogenic avian influenza viruses (HPAIV)[14].

Since 2003, HPAIVs H5N1 have spread to 62 countries in Eurasia and Africa and seriously affected poultry in Asia. Over 400 million birds have died from the infection or been killed for control purposes. A HPAIV is generated when a non-pathogenic virus brought in by migratory birds from nesting lakes in the north is transmitted to chickens via domestic ducks, geese, quails, turkeys and acquires pathogenicity for chickens. During over-wintering, some migratory birds were conversely infected with HPAIV H5N1 from poultry and have been found dead at lakes in northern China, Mongolia, Japan, Russia, Europe and Africa in April to May on the way back to their nesting lakes in northern territories. It was found that each of the viruses isolated from these birds were genetically closely related to those of the isolates from poultry in China[2,10,16,17]. Thus HPAIV strains that are currently circulating in poultry have returned to migratory water birds and spread world wide[10].

Since it is of concern that this H5N1 virus may perpetuate in the lakes in Siberia where migratory ducks nest in summer, virological surveillance and phylogenetic analysis of influenza viruses have been carried out in autumn when these birds flew to Hokkaido, Japan in 2008–2009.

It is known that the PB2 protein is a component of the viral polymerase complex that plays an important role in virus replication[5,11,19], and is a determinant of host range and pathogenicity of influenza viruses[18,20]. Therefore, PB2 genes of influenza viruses isolated from migratory ducks have been phylogenetically analyzed in the present study.

Materials and Methods

Sample collection and virus isolation: A total of 1,626 fecal samples of wild water birds were collected in autumn in 2008–2009 from Lake Ohnuma, Wakkanai, and Ohno pond, Hokkaido University, Sapporo, Japan. The fecal samples collected were kept in chilled containers and transported to our laboratory. Virus isolation and subtyping were performed as previously described[9]. One virus of each of the HA and NA combinations was selected randomly by year of isolation for genetic analyses (Table 1).

RNA extraction, RT-PCR, and nucleotide sequencing: RNA extraction and RT-PCR were conducted as previously described[13]. Partial-length PB2 genes were amplified using PB2 gene-specific primer set PB2-625F (5'-CAT GTA TGC TAC CAT CAA GGG-3'), and the universal primer Ba-PB2-2341R[6]. The PCR products were separated by 0.8% agarose gel electrophoresis and purified using the MiniElute™ Gel Extraction Kit (Qiagen, USA) as recommended by the manufacturer. The purified products were used as templates in sequencing reactions using a BigDye terminator cycle sequencing ready reaction kit and analyzed on a 3130 Genetic Analyzer (Applied Biosystems). DNA sequences were assembled and edited using the program Genetyx ATGC (2008 Genetyx Corp.). The accession numbers of PB2 genes sequenced in this study are available from DDBJ/EMBL/GenBank under accession numbers given in Table 2.

Phylogenetic analysis of the PB2 genes: Phylogenetic analysis was conducted using PB2 gene sequences of 36 representative strains from a total of 54 that were sequenced. Published sequences used in this study for phylogenetic comparison were obtained using BLAST homology searches from the influenza sequence database (http://www.ncbi.nlm.nih.gov/genomes/FLU/FLU.html). The PB2 gene tree was generated using the Neighbor Joining (NJ) bootstrap method (1,000 replicates) implemented in the Molecular Evolutionary Genetics Analysis program (MEGA, version 4.0)[22]. The evolutionary distances were calculated by the Maximum Composite Likelihood method[21].

Results

Influenza A viruses isolated from fecal samples of wild water birds flying from their nesting lakes in Siberia to Hokkaido, Japan in autumn

In the surveillance of avian influenza conducted in Hokkaido in autumn 2008–2009, 62 influenza viruses have been isolated from a total of 1,626 fecal samples. The HA (H1, H3-H7, H9-H12) and NA (N1-N3, N5-N9) subtypes of the isolates were identified. Twenty one HA and NA combinations were detected in the present study (Table 1). In the surveillance studies in 2000–2009 performed by our laboratory, no H5N1 HPAIV was isolated from wild water birds that flew from their nesting lakes in Siberia to Hokkaido, Japan in autumn[13].

Sequencing and phylogenetic analysis of the PB2 genes of influenza virus isolates from migratory birds

Randomly selected 54 isolates out of 283 avian influenza viruses isolated in the surveillance studies in 2000–2009 were sequenced of which 36 were phylogenetically analyzed. A phylogenetic tree was constructed on the basis of the partial nucleotide sequences of the PB2 genes (positions 1425–2192) of viruses isolated from wild water birds in Hokkaido in 2000 to 2009 (Fig. 1).

Table 1. Influenza viruses isolated from fecal samples of free-flying water birds 2008–2009

Subtypes of influenza viruses isolated in following years	
2008	2009
H3N2 (1)[a]	H1N3 (1)
H3N6 (3)	H1N5 (1)
H4N6 (11)	H4N6 (5)
H5N2 (1)	H5N1 (1)
H6N1 (4)	H5N2 (1)
H6N2 (1)	H6N1 (4)
H6N5 (1)	H6N8 (2)
H6N8 (1)	H11N9 (3)
H6N9 (1)	H12N5 (1)
H7N7 (1)	
H9N5 (1)	
H9N9 (1)	
H10N9 (2)	
H10N7 (11)	
H11N9 (2)	
H12N2 (1)	

[a]Number of isolates of subtypes were shown in parenthesis.

Phylogenetic tree of the PB2 genes was divided into American and Eurasian lineages. Duan *et al.*[3] showed that Eurasian lineage could be further divided into early and contemporary sublineages. Phylogenetic analysis of the PB2 genes of the isolates in the present study, belonged to the Eurasian lineage and were grouped (bootstrap values more than 85) into contemporary sublineages I and II. All the viruses analyzed in the present study belonged to either contemporary sublineage I or sublineage II. The majority of the PB2 genes under study clustered in different groups of sublineage I. They either clustered together or showed close relation to the PB2 genes of influenza viruses isolated from domestic and wild birds in China, Russia, Australia and Korea. It was noted that some of the strains, A/duck/Hokkaido/WZ76/2008

Table 2. Influenza virus strains analyzed in this study

Virus strain[a]	Subtype	Accession number	Virus strain	Subtype	Accession number
A/duck/Hokkaido/379/00	H4N6	AB478622	A/duck/Hokkaido/277/06	H6N2	AB478604
A/duck/Hokkaido/69/00	H5N3	AB300036	A/duck/Hokkaido/W162/06	H6N5	AB478618
A/duck/Hokkaido/18//00	H10N4	AB282876	A/duck/Hokkaido/W299/06	H9N2	AB478621
A/duck/Hokkaido/1169/01	H1N1	AB478607	A/duck/Hokkaido/W95/06	H10N8	AB569460
A/duck/Hokkaido/95/01	H2N2	AY422042	A/duck/Hokkaido/W73/07	H1N1	AB478614
A/duck/Hokkaido/17/01	H2N3	AY422040	A/duck/Hokkaido/W282/07	H4N6	AB478623
A/duck/Hokkaido/86/01	H2N3	AY422041	A/duck/Hokkaido/167/07	H5N3	AB378679
A/duck/Hokkaido/1005/01	H3N6	AB478606	A/duck/Hokkaido/201/07	H5N3	AB378687
A/duck/Hokkaido/56/01	H3N8	AB478611	A/duck/Hokkaido/69/07	H8N4	AB569464
A/duck/Hokkaido/1058/01	H4N5	AB569458	A/duck/Hokkaido/75/08	H3N6	AB569452
A/duck/Hokkaido/1019/01	H4N6	AB569457	A/duck/Hokkaido/W79/08	H4N6	AB569462
A/duck/Hokkaido/24/02	H11N9	AB478596	A/duck/Hokkaido/69/08	H4N6	AB569448
A/duck/Hokkaido/83/04	H1N1	AB478598	A/duck/Hokkaido/WZ21/08	H5N2	AB569454
A/duck/Hokkaido/18/04	H3N8	AB478595	A/duck/Hokkaido/W67/08	H6N1	AB569588
A/duck/Hokkaido/143/04	H4N2	AB569459	A/duck/Hokkaido/WZ76/08	H6N2	AB569453
A/duck/Hokkaido/W5/04	H4N6	AB569461	A/duck/Hokkaido/W112/08	H6N5	AB569466
A/duck/Hokkaido/193/04	H5N3	AB299377	A/duck/Hokkaido/W54/08	H6N8	AB569449
A/duck/Hokkaido/257/04	H6N1	AB478601	A/duck/Hokkaido/W76/08	H6N9	AB569465
A/duck/Hokkaido/W109/04	H6N2	AB478616	A/duck/Hokkaido/229/08	H7N7	AB569456
A/duck/Hokkaido/W12/04	H6N2	AB478609	A/duck/Hokkaido/238/08	H9N2	AB569467
A/duck/Hokkaido/W59/04	H8N4	AB478612	A/duck/Hokkaido/131/08	H10N7	AB569451
A/duck/Hokkaido/89/04	H10N5	AB478599	A/duck/Hokkaido/WZ16/08	H10N9	AB569463
A/duck/Hokkaido/W259/05	H2N5	AB478620	A/duck/Hokkaido/W45/08	H11N9	AB569455
A/duck/Hokkaido/12/05	H3N2	AB478594	A/ws/Hokkaido/OIE110/08	H12N2	AB569450
A/duck/Hokkaido/W70/05	H3N8	AB478613	A/duck/Hokkaido/WZ75/09	H5N2	AB569468
A/duck/Hokkaido/W268/05	H6N1	AB478603			
A/duck/Hokkaido/260/05	H8N4	AB478602			
A/duck/Hokkaido/279/06	H4N6	AB478605			
A/duck/Hokkaido/W206/06	H6N1	AB478619			

[a]Name of the virus with corresponding accession number of PB2 genes sequenced in this study.

(H6N2), A/duck/Hokkaido/W76/2008 (H6N9) and A/duck/Hokkaido/69/2008 (H4N6) characterized in this study were closely related to strains A/mallard/Korea/gH170/2007 (H7N7) and A/magpie/Korea/YJDI74/2007 (H7N7) isolated from domestic birds in Korea. Some viruses that fell in sublineage I were phylogenetically closely related to an isolate obtained from pintails in Alaska, virus strain A/northernpintail/Alaska/44204-108/06 (H3N1). Novel reassortant H5N1 HPAIV, A/chicken/Laos/P0130/2007 (H5N1) isolated from Laos[1)] also belonged to this sublineage but was most closely related to a virus isolated in migratory birds in Korea, virus strain A/shorebird/Korea/S6/2006 (H1N2). The Eurasian sublineage II consisted of only one group (Fig 1). The H5N1

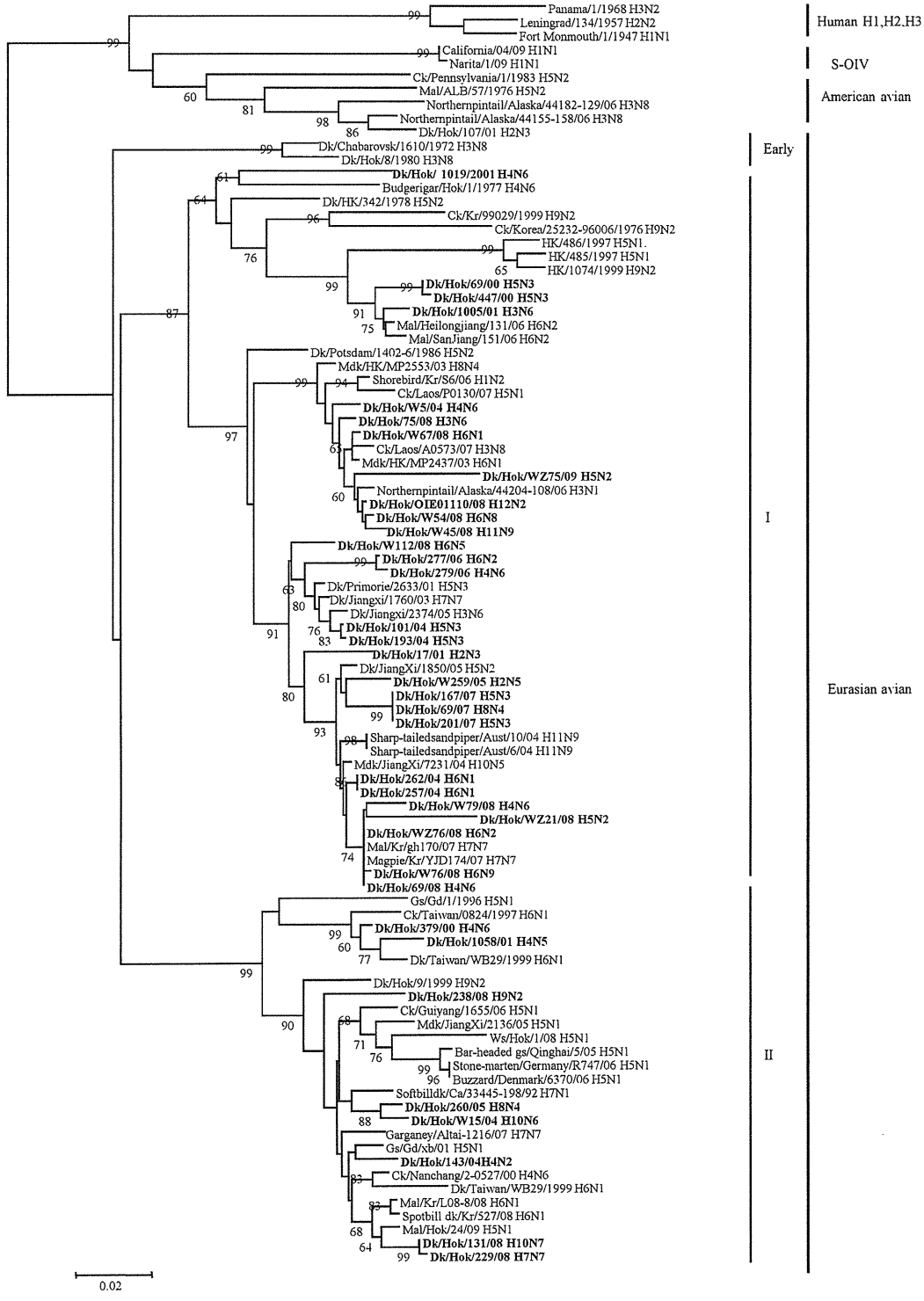

Fig. 1. Phylogenetic tree of influenza A virus PB2 genes. The phylogenetic tree was constructed using neighbour joining (NJ) method (1,000 replicates). For construction of this tree, 36 representative strains from a total of 54 that were sequenced. PB2 gene sequences each comprising 767 nucleotides (positions 1425–2192) were analyzed. This figure showing complete phylogram of avian influenza virus lineages with overall lineage of these isolates were of Eurasian avian and divided further into 2 distinct contemporary sublineages I and II. Bootstrap values below 60 are not shown. The strains sequenced in this study are indicated in bold.

HPAIV isolated from wild birds in China, Europe, and Japan belonged to this sublineage but none of the isolates tested in this study are closely related to these H5N1 HPAIVs.

Discussion

Rapid world wide spread of HPAIV to 62 countries in Eurasia and Africa with H5N1 viruses isolated from water birds found dead in Mongolia on the way back to their nesting lakes in Siberia in spring 2005, 2006, 2009 and 2010 raises concern that they may perpetuate in the northern nesting lakes in Siberia in summer. Since it was found that these H5N1 HPAIVs were genetically closely related to those influenza viruses isolated from birds in China, Iraq, Croatia, Nigeria, Korea and Japan, intensive surveillance of avian influenza in migratory water birds needs to be continued. In 2008–2009 avian influenza surveillance, we isolated 62 influenza viruses from fecal samples collected from migratory ducks that flew from their northern nesting lakes to Hokkaido, Japan in autumn. Influenza viruses of different subtypes have been isolated from these wild water birds. Twenty one combinations of the HA and NA subtypes of influenza viruses were detected. No H5N1 HPAIV was found during the surveillance period, indicating that the H5N1 HPAIV has not been perpetuated, at least dominantly in wild water birds that nest in northern territory in summer. The present findings are in agreement with previous study[13] showing that the H5N1 HPAIV has not persisted yet in wild water birds that nest in Siberia in summer.

The phylogenetic analyses in the present study revealed that none of the PB2 gene sequences of influenza viruses tested were closely related to HPAIV and none belonged to the American lineage. However, previous studies conducted in our laboratory found some internal protein genes (PB2, PA, and M) of influenza viruses isolated from migratory birds in Hokkaido which phylogenetically clustered with those of influenza viruses of the American lineage[12,13], indicating that interregional transmission of influenza virus genes do occur between the American and Eurasian gene pools among viruses obtained in Hokkaido. The grouping together of the PB2 gene of an influenza virus isolated from a pintail *(Anas acuta)* in Alaska with those of some viruses examined presently testifies to this phenomenon. The pintail (*Anas acuta*) species has been implicated in the inter-hemispheric transmission of influenza viruses between the American and Eurasian gene pools[8].

In conclusion, intensive surveillance of avian influenza conducted in Hokkaido in autumn in 2008–2009, has demonstrated that no HPAIVs were isolated from wild water birds flying from their nesting lakes in Siberia, indicating that the HPAIV has not yet persisted in their nesting lakes where they nest in summer. However, there is no guarantee that the absence of H5N1 HPAIV in wild water birds that come to Hokkaido is a permanent status. Therefore, the present study highlights the need for continued surveillance of avian influenza viruses in wild and domestic birds for the prevention and control of influenza.

Acknowledgements

We extend special thanks to members of the Wakkanai bird house, Laboratory of Microbiology and Department of Global Epidemiology for helping in the samples collection and providing technical support respectively. The present work is supported by the Program of Founding Research Centers for Emerging and Reemerging Infectious Disease from the Ministry of Education, Culture, Sports, Science, and Technology, and SORST Japan.

References

1) Boltz, D. A., Douangngeun, B., Phommachcnh, P., Sinthasak, S., Mondry, R., Obert, C., Seiler, P., Keating, R., Suzuki, Y., Hiramatsu, H., Govorkova, E. A., and Webster, R. G. 2010. Emergence of H5N1 avian influenza viruses with reduced sensitivity to neuramidase inhibitors and novel reassortants in Lao People's Democratic Republic. *J. Gen. Virol.,* **91**: 945-959.
2) Chen, H., Li, Y., Li, Z., Shi, J., Shinya, K., Deng, G., Qi, Q., Tian, G., Fan, S., Zhao, H., Sun, Y., and Kawaoka, Y. 2006. Properties and dissemination of H5N1 viruses isolated during an influenza outbreak in migratory waterfowl in western China. *J. Virol.,* **80**: 5976-5983.
3) Duan, L., Campitelli, L., Fan, X. H., Leung, Y. H., Vijaykrishnan, D., Zhnag, J. X., Donatelli, I., Delogu, M., Li, K. S., Foni, E., Chiapponi, C., Wu, W. L., Kai, H., Webster, R. G., Shortridge, K. F., Peiris, J. S. M., Smith, G. J. D., Chen, H., and Guan, Y. 2007. Characterization of low-pathogenic H5 subtype influenza viruses from Eurasia: implications for the origin of highly pathogenic H5N1 viruses. *J. Virol.*, **81**: 7529-7539.
4) Fouchier, R. A., Munster, V., Wallensten, A., Bestebroer, T. M., Herfst, S., Smith, D., Rimmelzwaan, G. F., Olsen, B., Osterhaus, A. D. 2005. Characterization of a novel influenza A virus hemagglutinin subtype (H16) obtained from black-headed gulls. *J. Virol.,* **79**: 2814-2822.
5) Hatta, M., Gao, P., Halfmann, P., Kawaoka, Y. 2001. Molecular basis for high virulence of Hong Kong H5N1 influenza A viruses. *Science,* **293**: 1840-1842.
6) Hoffman, E., Stech, J., Guan, Y., Webster, R. G. and Perez, D. R. 2001. Universal primer set for the full-length amplification of all influenza A viruses. *Arch. Virol.,* **146**: 2275-2289.
7) Ito, T., Okazaki, K., Kawaoka, Y., Takada, A., Webster, R. G., Kida, H. 1995. Perpetuation of influenza A viruses in Alaskan waterfowl reservoirs. *Arch. Virol.,* **140**: 1163-1172.
8) Jahangir, A., Watanabe, Y., Chinen, O., Yamazaki, S., Sakai, K., Okamura, M., Nakamura, M., and Takehara, K. 2008. Surveillance of avian influenza viruses in Northern pintails (Anas acuta) in Tohoku district, Japan. *Avian Dis.,* **52**: 49-53.
9) Kida, H., Yanagawa, R. 1979. Isolation and characterization of influenza A viruses from wild free-flying ducks in Hokkaido, Japan. *Zentralbl. Bakteriol. Orig. A.,* **244**: 135-143.
10) Kida, H. 2008. Ecology of Influenza Viruses in Nature, Birds, and Humans. *Global Environmental Research. Assoc. of International Research Initiatives for Environmental Studies.,* **12**: 9-14.
11) Li, K. S., Guan, Y., Wang, J., Smith, G. J. D., Xu, K. M., Duan, L., Rahardjo, A., Puthavathana, P., Buranathai, C., Nguyen, T. D., Estoepangestie, A. T. S., Chaisingh, A., Auewaraku, P., Long, H. T., Hanh, N. T. H., Webby, R. J., Poon, L. L. M., Chen, H., Shortridge, K. F., Yuen, K. Y., Webster, R. G., and Peiris, J. S. M. 2004. Genesis of a highly pathogenic and potentially pandemic H5N1 influenza virus in eastern Asia. *Nature,* **430**: 209-213.
12) Liu, J. H., Okazaki, K., Bai, G. R., Shi, W. M., Mweene, A., and Kida, H. 2004. Interregional transmission of the internal protein genes of H2 influenza virus in migratory ducks from North America to Eurasia. *Virus Genes,* **29**: 81-86.
13) Manzoor, R., Sakoda, Y., Mweene, A., Tsuda, Y., Kishida, N., Gui-Rong, Bai, G. R., Kameyama, K. I., Isoda, N., Soda, K., Naito, M., Kida, H. 2008. Phylogenetic analysis of the M genes of influenza viruses isolated from free-flying water birds from their northern territory to Hokkaido, Japan. *Virus Genes,* **37**: 144-152.
14) Munster, V. J., Wallensten, A., Baas, C., Rimmelzwaan, G. F., Schutten, M., Olsen, B., Osterhaus, A. D., and Fouchier, R. A. 2005. Mallards and highly pathogenic avian influenza ancestral viruses, northern Europe. *Emerg. Infect. Dis.,* **11**: 1545-1551.
15) Okazaki, K., Takada, A., Ito, T., Imai, M., Takakuwa, H., Hatta, M., Ozaki, H., Tanizaki, T., Nagano, T., Ninomiya, A., Demenev, V. A., Tyaptirganov, M. M., Karatayeva, T. D., Yamnikova, S. S., Lvov, D. K., and Kida, H. 2000. Precursor genes of future pandemic influenza viruses are perpetuated in ducks nesting in Siberia. *Arch. Virol.*, **145**: 885-893.
16) Peiris, M. J. S., de Jong, M. D., Guan, Y. 2007. Avian influenza virus (H5N1): A threat to human health. *Clin. Microbiol. Rev.,* **20, 2**: 243-267.
17) Sakoda, Y., Sugar, S., Batchluun, D., Erdene-Ochir, T. O., Okamatsu, M., Isoda, N., Soda, K., Takakuwa, H., Tsuda, Y., Yamamoto,

N., Kishida, N., Matsuno, K., Nakayama, E., Kajihara, M., Yokoyama, A., Takada, A., Sodnomdarjaa, R. and Kida, H. 2010. Characterization of H5N1 highly pathogenic avian influenza virus strains isolated from migratory waterfowl in Mongolia on the way back from the southern Asia to their northern territory. *Virology*, **406**: 88–94.

18) Smith, G. J., Naipospos, T. S., Nguyen, T. D., de Jong, M. D., Vijaykrishna, D., Usman, T. B., Hassan, S. S., Nguyen, T. V., Dao, T. V., Bui, N. A., Leung, Y. H., Cheung, C. L., Rayner, J. M., Zhang, J. X., Zhang, L. J., Poon, L. L., Li Nguyen, V. C., Hien, T. T., Farrar, J., Webster, R. G., Chen, H., Peiris, J. S., Guan, Y. 2006. Evolution and adaptation of H5N1 influenza virus in avian and human hosts in Indonesia and Vietnam. *Virology,* **350**: 258–268.

19) Subbarao, K., London, W., and Murphy, B. R. 1993. A single amino acid in the PB2 gene of influenza A virus is a determinant of host range. *J.Virol.,* **67**: 1761–1764.

20) Subbarao, K., Shaw, M. W. 2000. Molecular aspects of avian influenza (H5N1) viruses isolated from humans. *Rev. Med. Virol.,* **10**: 337–348.

21) Tamura, K., Nei, M., Kumar, S. 2004. Prospects for inferring very large phylogenies by using the neighbor-joining method. *Proc. Natl. Acad. Sci. U. S. A.,* **101**: 11030.

22) Tamura, K., Dudley, J., Nei, M., Kumar, S. 2007. MEGA4: Molecular evolutionary genetics analysis (MEGA) software version 4.0. *Mol. Biol. Evol.*, **24**: 1596–1599.

23) Webster, R. G., Bean, W. J., Gorman, O. T., Chambers, T. M. and Kawaoka, Y. 1992. Evolution and ecology of influenza A viruses. *Microbiol. Rev.*, **56**: 152–179.

Japanese Journal of Veterinary Research 59(1): 23-29, 2011

FULL PAPER

A vaccine prepared from a non-pathogenic H5N1 influenza virus strain from the influenza virus library conferred protective immunity to chickens against the challenge with antigenically drifted highly pathogenic avian influenza virus

Rozanah Asmah Abdul Samad[1)], Naoki Nomura[1)], Yoshimi Tsuda[1)], Rashid Manzoor[2)], Masahiro Kajihara[2)], Daisuke Tomabechi[2)], Takashi Sasaki[3)], Norihide Kokumai[3)], Toshiaki Ohgitani[3)], Masatoshi Okamatsu[1, 4)], Ayato Takada[2)], Yoshihiro Sakoda[1, 4)] and Hiroshi Kida[1, 4, 5, 6*)]

[1)]Laboratory of Microbiology, Department of Disease Control, Graduate School of Veterinary Medicine, Hokkaido University, Sapporo 060-0818, Japan

[2)]Department of Global Epidemiology, Hokkaido University Research Center for Zoonosis Control, Sapporo 001-0020, Japan

[3)]Avian Biologics Department, Kyoto Biken Laboratories, Inc., Uji, Kyoto, 611-0041, Japan

[4)]OIE Reference Laboratory for Highly Pathogenic Avian Influenza, Sapporo 060-0818, Japan

[5)]Hokkaido University Research Center for Zoonosis Control, Sapporo 001-0020, Japan

[6)]SORST, Japan Science and Technology Agency (JST), 4-1-8 Honcho Kawaguchi, Saitama 332-0012, Japan

Received for publication, November 24, 2010; accepted, December 14, 2010

Abstract

Inactivated influenza virus vaccine prepared from a non-pathogenic influenza virus strain A/duck/Hokkaido/Vac-1/2004 (H5N1) from the virus library conferred protective immunity to chickens against the challenge of antigenically drifted highly pathogenic avian influenza virus (HPAIV), A/whooper swan/Hokkaido/1/2008 (H5N1). The efficacy of the vaccine was comparable to that prepared from genetically modified HPAIV strain ΔRRRRK rg-A/whooper swan/Mongolia/3/2005 (H5N1), which is more antigenically related to the challenge virus strain, in chickens.

Keywords: *Antigenically drifted HPAIV, vaccine*

*Corresponding author: Hiroshi Kida, Laboratory of Microbiology, Department of Disease Control, Graduate School of Veterinary Medicine, Hokkaido University, Sapporo 060-0818, Japan
Phone: +81-11-706-5207. Fax: +81-11-706-5273. E-mail: kida@vetmed.hokudai.ac.jp

Introduction

H5N1 highly pathogenic avian influenza virus (HPAIV) is causing panzootic outbreaks in poultry in Eurasia and Africa, posing serious concern for public health as well as live stock industry. The outbreaks of highly pathogenic avian influenza caused by H5N1 viruses that spread to 62 countries have taken toll of 300 million poultry (dead or been killed) and 15 countries with human fatalities[5]. In addition, the H5N1 HPAIVs had returned to migratory birds, spread to Eurasia and Africa[2,6]. Since each of the hemagglutinin (HA) genes of pandemic influenza viruses has been originated from avian influenza viruses[14], is now a potential pandemic threat. H5N1 viruses isolated from water birds found dead in Mongolia on the way back to their nesting lakes in Siberia in April to May in 2005, 2006, 2009 and 2010 were genetically closely related to H5N1 viruses isolated from birds in China, Iraq, Croatia, Nigeria, Korea and Japan[2,6,10]. Viruses similar to those have spread world-wide and it is therefore, a serious concern that these HPAIVs persists in Eurasia may perpetuate in the lakes where they nest in summer and that those birds may bring HPAIVs to the south in autumn.

Stamping-out and movement restriction are the standard measures for the control of highly pathogenic avian influenza (HPAI) in poultry and found to be successful in rapid eradication of the HPAIV infection[4]. Vaccination is a limited application as an optional tool when stamping-out is not effective enough to control the disease[1]. Vaccination may be an optional measure in cases where the disease spread widely. Many commercial vaccines have been prepared from viruses of the North American lineage. These vaccines may be less effective for the control of current HPAI outbreaks caused by the infection with viruses of the Eurasian lineage in Asia[5]. Inactivated influenza vaccines for the control of the circulating avian influenza particularly in Asia, therefore, should be prepared from an H5N1 virus strain belonging to the Eurasian lineage.

The OIE Reference Laboratory for HPAI at Hokkaido University has established the library of influenza viruses of all HA and neuraminidase (NA) subtypes and their genes[6]. The previous study[12] has demonstrated that the library of a panel of influenza virus strains isolated from natural hosts is useful for the preparedness for future pandemics. These influenza virus strains are stored in the library and have been used for the purpose of vaccine production and diagnosis.

Prolonged endemics of H5N1 virus in poultry has been known to generate antigenically and genetically diversified viruses in some countries[9]. Previous study[5,8], showed that avian influenza vaccine prepared from non-pathogenic avian influenza viruses from the library conferred protective immunity against the challenge virus of antigenically similar. Ideally, vaccine strains that are antigenically and genetically closely related to the circulating variant strain and induce immunity against antigenically drifted virus are preferable. In the present study, the efficacy of the vaccine prepared from a non-pathogenic influenza virus strain A/duck/Hokkaido/Vac-1/2004 (H5N1) from the virus library was comparable to that prepared from genetically modified HPAIV strain ΔRRRRK rg-Mon/05 (H5N1) by reverse genetics against the challenge with antigenically drifted HPAIV, A/whooper swan/Hokkaido/1/2008 (H5N1) in chickens.

Materials and Methods

Viruses: Influenza viruses, A/whooper swan/Hokkaido/1/2008 (H5N1) (Ws/Hok/08), A/duck/Hokkaido/Vac-1/2004 (H5N1) (Dk/Vac-1/04) and A/whooper swan/Mongolia/3/2005 (H5N1) (Ws/Mon/05) and mutant A/whooper swan/Mongolia/

3/2005 (H5N1) (ΔRRRRK rg-Mon/05), of clade 2.3.2, classical and clade 2.2 respectively were used. All viruses used in this study have been propagated and characterized antigenically and genetically as described[9]. All viruses were propagated in 10-day-old embryonated chicken eggs at 35°C for 30 to 48 hrs and stored at −80°C until use.

Dk/Vac-1/04 and ΔRRRRK rg-Mon/05 were used for vaccine preparation. A non-pathogenic avian influenza Dk/Vac-1/04 virus from the library, was generated as a reassortant virus between A/duck/Mongolia/54/2001 (H5N2) and A/duck/Mongolia/47/2001 (H7N1)[5]. Ws/Mon/05 virus isolated from a whooper swan (*Cygnus Cygnus*) found dead in Lake Khunt nuur, Mongolia[10] was genetically modified by reverse genetics with site-directed mutagenesis to generate a mutant ΔRRRRK rg-Mon/05 strain.

Meanwhile, Ws/Hok/08 (H5N1) virus that was isolated from whooper swan found dead at Notsuke Peninsular, in Hokkaido, Japan in May on their way back to their nesting lakes in northern territories[9] was used as the challenge virus.

Preparation of a genetically modified H5N1 HPAIV: Ws/Mon/05 was genetically modified by reverse genetics with site-directed mutagenesis as described[7]. Briefly, the amino acid RRRRK at the cleavage site of the HA were deleted and replaced with amino acid T by site-directed mutagenesis. The T mutation was introduced into the HA of Ws/Mon/05 cloned into the pHW2000 expression vector, using a Quick Change site-directed mutagenesis kit (Stratagene) according to the manufacturer's instructions. The presence of the desired mutations was confirmed by sequencing the full length of the cloned HA genes. The mutant virus was designated ΔRRRRK rg-Mon/05 and confirmed to be apathogenic to chickens.

Intravenous pathogenicity (IVPI) of ΔRRRRK rg-Mon/05 in chickens: The intravenous pathogenicity index of ΔRRRRK rg-Mon/05 was carried out according to the OIE standard method[8]. Briefly, 1/10 dilutions of infectious allantoic fluids containing the viruses were intravenously inoculated into eight 6-week-old chickens. These chickens were housed for 10 days in self-contained isolator units (Tokiwa Kagaku Kikai Co., Ltd., Tokyo) at a BSL 3 facility at the Hokkaido University Research Center for Zoonosis Control, Japan. All animal experiments were conducted in accordance to guidelines of the Institutional Animal Care and Use Committee of Hokkaido University, Japan.

Vaccine preparation: A virus suspension of Dk/Vac-1/04 and ΔRRRRK rg-Mon/05 were inactivated by incubation with formalin at a 0.2% final concentration for 3 days at 4°C respectively. Virus inactivation was confirmed by inoculation into embryonated chicken eggs. The inactivated Dk/Vac-1/04 and ΔRRRRK rg-Mon/05 viruses suspension were diluted with phosphate-buffered saline (pH = 7.2) (PBS) to appropriate concentrations based on HA titers and adjuvanted as described[5,11].

Potency test of vaccine efficacy in chickens against antigenically drifted strains Ws/Hok/08: The potency of the vaccines was evaluated by challenging chickens inoculated with vaccines prepared from Dk/Vac-1/04 or ΔRRRRK rg-Mon/05 with antigenically drifted Ws/Hok/08. Thirty 4-week-old chickens were divided into three groups and the inactivated avian influenza virus Dk/Vac-1/04 or ΔRRRRK rg-Mon/05 vaccines were intramuscularly inoculated to ten chickens respectively as described[5,11]. PBS was inoculated in ten control chickens. Three weeks after vaccination, all chickens were challenged intranasally with a dose 10^3 50% chicken lethal dose (CLD_{50}) of Ws/Hok/08. Four chickens of each group were sacrificed on day 3 post-challenge and the remaining six chickens were observed